Welwitschia mirabilis – induction, growth and organization of mature leaf callus

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Welwitschia mirabilis is a phylogenetically important primitive gymnosperm of the monogeneric family Welwitschiaceae. The plant is unique in appearance, having only two leaves throughout its life span. It is a long-lived, slow growing plant and could prove as an excellent experimental material for the study of anti-ageing gene. Because of non-availability of plant material and the intractable nature of the plant, limited tissue culture work has been conducted. In the present study, callus was induced from mature leaf-tip explants from a 26-year-old plant. The induced callus grows at a comparatively faster pace. Although successful induction of organized shiny and smooth, globular structures were observed, no shoots had developed in the callus.

Keywords: Callus induction, leaf-tip explants, living fossil, *Welwitschia mirabilis*.

THE genus *Welwitschia* is endemic to the Namib Desert, one of the world's driest regions where it was discovered by Austrian botanist Friedrich Welwitsch during 1860 and after whom the plant has been named. *Welwitschia mirabilis* is a monotypic genus of a monogeneric family (Welwitschiaceae). Morphologically, it is most unique in the plant kingdom, both living and fossil, and can be called an 'adult seedling'. The plant seems 'monster-like', most likely to have originated on another planet. It is unusual for its large, strap-like leaves that grow continuously over 500 years along the ground. During its entire life, each plant produces only two leaves, which often split into many being whipped by the winds. [14]C dating of the largest plants has shown that some individuals were over 1500 years old. Its short, woody, unbranched stem that grows from a basal meristem becomes twisted and frayed with the passing centuries.

Welwitschia has been classified as a primitive gymnosperm, a dioecious member of the order Gnetales, but scientists have determined that the vascular tissue (xylem) was typical of that found in the flowering plants, or angiosperms[1]. The plants are protected by law in their native habitat. The important phylogenetic position, odd morphology, and unique ecological adaptations make this an important plant to be studied from an evolutionary as well as research and development viewpoint. Normally,

W. mirabilis is propagated by seeds. Since it was included in CITES Appendix II-2007 and 14 September 2014 (http://www.cites.org/sites/default/files/eng/app/2014/E-Appendices-2014-09-14.pdf), a production of seed culture could also reduce pressure on wild populations, and in the long term could produce plants for planting in the original habitat. Seeds were produced on cultivated plants, but the success rate was unreasonably very low[2,3].

There are limited reports available on *W. mirabilis*, mainly because of rare availability of the experimental material and its slow growth nature. Khoshoo and Ahuja[4] reported chromosomal studies in root tips of the germinated seedlings of *W. mirabilis*. Jacobsen and Lester[5] conducted Cluster analysis and analysis of molecular variance (AMOVA), which revealed significant levels of variation within and between populations of *W. mirabilis*, with little evidence of inbreeding. Earlier report on tissue culture by Bornman[6] was on induction of callus from the hypocotyl–root axis of the germinating embryos of *W. mirabilis* which could be grown successfully, but unsuccessful in either inducing embryogenesis or organogenesis. Bornman and Fanshawe[7,8] have further reported the structural organization and responses of *W. mirabilis* callus to sucrose concentration (2–8%) and stress factors by increasing culture temperature and nutrient agar concentration respectively, but were unsuccessful with regard to organogenesis. In the present work, leaf tips were excised from a 26-year-old mature plant as against seedling explants used in the previous reports and efforts were made for inducing direct or indirect organogenesis or embryogenesis from leaf-segments. The use of leaf-tip explants did not cause any damage to the growing plant. Tissue culture of *W. mirabilis*, a pre-requisite to any genetic transformation work, will open new vistas for further studies on the genes related to longevity.

Material and methods

Plant material

The plants of *W. mirabilis* are precious as we have only one plant growing for the last 26 years in the Botanic Garden, CSIR-NBRI campus, Lucknow, India (Figure 1). The trunk of the plant is short with only two leaves that continuously grow from the base in opposite directions. Leaves are the only option to be used for explants. Leaf

tip of approximately 5 cm was cut from each leaf, washed thoroughly under running tap water for 15–20 min, then surface-disinfested with 5% Labolene (Qualigens) solution with a drop of Tween-20 for 5 min, followed by rinsing with distilled water. The explants were then rinsed with 70% alcohol for 30 sec, surface disinfested with 0.1% (v/v) $HgCl_2$ solution for 8 min with constant shaking, and rinsed thoroughly with sterilized distilled water 3–4 times. Small leaf segments measuring approximately 0.5–1 cm^2 were excised and cultured for callus induction in Murashige and Skoog[9] (MS) medium containing different combinations of 9, 22.5, or 45 μM 2,4-D (2,4-dichlorophenoxyacetic acid), 2.2 or 4.4 μM BA (benzyl adenine), 4.6 μM Kn (kinetin) and 5.4 μM NAA (α-naphthalene acetic acid). Five explants per treatment were oriented in a horizontal position on the surface of the media, and cultures were incubated in the dark at 25°C for 4–6 weeks.

The pH of all media was adjusted to 5.8 before adding 0.8% (w/v) agar (Qualigens) and autoclaved at 121°C for 20 min. Cultures were incubated at 25 ± 2°C under a 16 h photoperiod, 8 h dark at 50–60 μmol m^{-2} s^{-1}. The cultures for callus induction were kept in continuous dark, whereas the cultures for organogenesis were kept under light and dark photoperiod.

Growth index analysis

To obtain the maximum growth of callus, the weight of the inoculum as well as the period of highest growth of the callus were analysed. Friable callus (1.5 or 2 g) were cultured on MS medium containing the optimum concentrations of plant growth regulators, 9.0 μM 2,4-D, 4.4 μM BA, 4.6 μM Kn along with 5.4 μM NAA in replicates of three and harvested individually. Fresh and dry weights of these calluses were recorded. Growth index (GI) of callus tissue was calculated at the end of 1 week up to 6 weeks using the formula

Growth index (GI) = Final fresh weight
– Initial fresh weight/initial fresh weight.

Figure 1. A 26-year-old plant of *Welwitschia mirabilis* growing in the Botanic Garden of CSIR-NBRI campus in Lucknow.

Direct and indirect organogenesis

For direct organogenesis from leaf segments, explants were cultured on MS medium supplemented with 4.4 μM BA and 14.25 μM IAA (indole-3-acetic acid), 4.5 μM TDZ (thiadiazurone) and 14.25 μM IAA, 9.3 μM Kn and 28.5 μM IAA, or 9.1 μM Z (zeatin) and 28.5 μM IAA and cultured in the light for 4–6 weeks.

For indirect organogenesis, leaf explants that developed friable white callus in the presence of 9 or 22.5 μM 2,4-D, 4.4 μM BA, 4.6 μM Kn and 5.4 μM NAA were selected for induction of somatic embryos using 0, 0.45 or 0.9 μM 2,4-D and keeping the cultures in the dark. A pulse treatment of TDZ and IAA was conducted with white friable callus, where 11.25 or 22.5 μM TDZ plus 5.7 μM IAA was used initially for 7–10 days followed by subculture to either 1.1 or 2.2 μM BA with 0.57 μM IAA or 1.1 μM TDZ with 0.57 μM IAA.

In another experiment, 45 μM 2,4-D and 4.7 μM Kn with 60 μM $AgNO_3$ and 0.25 μM PEG (polyethylene glycol) were used for organogenesis from callus. The developed friable callus was also given a pulse treatment of 4.0 μM TIBA (triiodobenzoic acid) initially for 10 days followed by subculture to 0.2 μM TIBA with 0.4 μM BA. Callus with small, rounded, globular, smooth structures was cultured on medium with 13.8 μM spermidine along with 1.2 μM Kn and 1.3 μM NAA. Later, 4.5 μM TDZ and 2.9 μM GA_3 (gibberellic acid) were also added to the medium to obtain organogenesis. The cultures were kept under light and dark photoperiod for 4–6 weeks.

Results and discussion

Callus induction

After 50–60 days, white friable callus developed at the cut margins of leaf segments when the explants started turning brown (Figure 2 *a* and *b*). Only the explants near the apical notch of the leaf responded and induced callus,
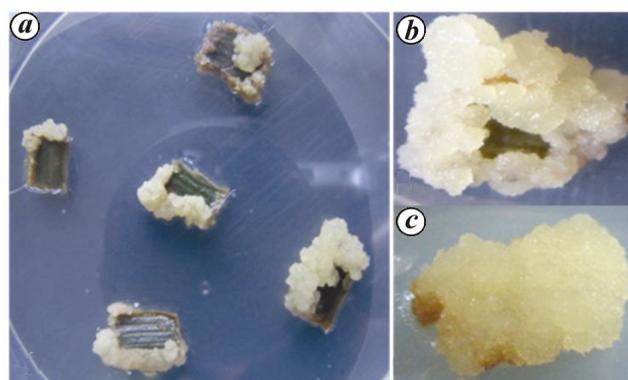

Figure 2. Callus induction from leaf explants under dark incubation. *a*, Callus after 6–8 weeks. *b*, Close-up view of callus after 8 weeks; *c*, Callus after 12 weeks of culture.
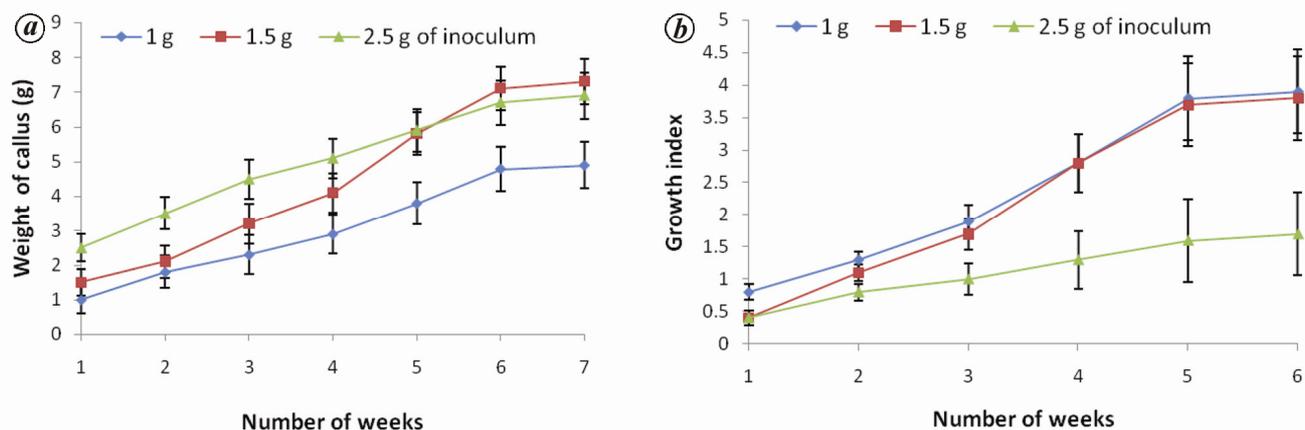
Figure 3. Growth kinetics of *W. mirabilis* callus cultures in MS medium supplemented with 9.0 μM 2,4-D, 4.4 μM BA, 4.6 μM Kn and 5.4 μM NAA over a culture period of 6 weeks. *a*, Fresh weight increase. *b*, Growth index. Bars represent mean ± standard error of three replications.

while all the other explants turned brown and necrosed. Leaf explants kept in the dark developed callus. MS medium with 9.0 μM 2, 4-D, 4.4 μM BA, 4.6 μM Kn and 5.4 μM NAA was the most effective optimum treatment for callus induction. Similar callus induction could be achieved with higher 2,4-D also, but the lowest concentration (9.0 μM) of 2,4-D was selected as the optimum. Growth was fast and within 12 weeks the leaf explants were covered with white friable callus (Figure 2 *c*). The earlier work of Bornman[6] reported induction of callus from the hypocotyl–root axis of germinating embryos from seedlings of *W. mirabilis*, whereas in the present work, the callus was induced from leaf-tip explants of 26-year-old mature plant. After every 6 weeks, callus was subcultured to fresh medium for further growth. Dark conditions were necessary for induction and growth of the callus, as under light the callus turned brown.

Growth behaviour of callus

White friable callus of *W. mirabilis* showed a sigmoid growth pattern and grew fast at all the three inoculum weights (1, 1.5 and 2 g). Weight of callus increased similarly in 1.0 (4.8-fold) and 1.5 g (4.73-fold) of inoculums, whereas 2.0 g inoculums grew (2.68-fold) slowly resulting in less increase in weight (Figure 3 *a*). The growth index and fresh weight of callus increased gradually from the first week of culture until the fifth week (Figure 3). White friable callus on the surface became dark after the fifth week onwards. Maximum growth occurred between 3–5 weeks. All calluses became dark in colour and growth ceased at the end of the seventh week of culture period. Browning of the culture medium was also observed at the end of the seventh week. Therefore, regular subcultures were done at 4-week intervals to a fresh medium to produce stable callus cultures and maintain callus viability.

Direct organogenesis

Leaf explants cultured on medium containing either of BA, TDZ, Kn or Z along with IAA (Table 1), turned brown without any response. In another experiment, the explants were treated initially with higher doses of TDZ and after 1 week of incubation, subcultured to lower concentration of TDZ or BA (Table 1), but differentiation of shoots could not be achieved. Although few explants induced callus in the presence of 4.5 μM TDZ, neither did the callus develop further nor did it regenerate shoots; instead the explants turned brown in the light.

Indirect organogenesis

In the first experiment, the callus developed in the presence of high concentration of 2,4-D was used to obtain somatic embryos (Figure 4 *a*). Honnale and Rao[10] reported 2,4-D as efficient auxin for somatic embryo induction. They observed that addition of BAP at low concentrations enhances the frequency and number of somatic embryos per explant in sesame. We have further subcultured the callus in the absence or lower concentration (0.45 μM) of 2,4-D, but supplemented with BA or TDZ along with 0.57 μM IAA. Although there was no differentiation of shoots, the white friable callus continued to grow.

In the second experiment, when either 60 μM $AgNO_3$ or 0.25 μM PEG individually or in combination was added to the medium containing 45 μM 2,4-D and 4.7 μM Kn, small, white, shiny, smooth and globular structures developed in the callus after 8 weeks of culture incubation in the dark (Figure 4 *b*). Later, the callus was subcultured to low concentration of these plant growth regulators, but these structures did not develop further. The entire callus turned brown when kept in the light. It was noted that the freshly developed callus was friable in texture, whereas small, rounded, smooth, white and

Table 1. Concentrations and combinations of plant growth regulators used for direct and indirect organogenesis from leaf callus of *Welwitschia mirabilis*

Initial treatment (µM)	Subculture (µM)	Callus response
*4.4 BA + 14.25 IAA	–	No response, except that explants from TDZ
*4.5 TDZ + 14.25 IAA		remained green but later turned brown
*9.3 Kn + 28.5 IAA		
*9.2 Z + 28.5 IAA		
**11.25 TDZ + 5.7 IAA	1.1 BA + 0.57 IAA	Turned brown
For 1 week	2.2 BA + 0.57 IAA	
	1.1 TDZ + 0.57 IAA	
**22.5 TDZ + 5.7 IAA	1.1 BA + 0.57 IAA	Turned brown
For 1 week	2.2 BA + 0.57 IAA	
	1.1 TDZ + 0.57 IAA	
**4 TIBA + 4.4 BA	0.2 TIBA + 0.4 BA	Turned brown
For 10 days		
**45 2,4-D + 60 AgNO$_3$ + 4.7 Kn	–	Small, rounded, globular, smooth structures developed
**45 2,4-D + 60 AgNO$_3$ + 4.7 Kn	0.45 2,4-D + 6 AgNO$_3$ + 0.5 Kn	White and friable
**45 2,4-D + 0.25 PEG + 4.7 Kn	–	Small, rounded, globular, smooth structures developed
**45 2,4-D + 0.25 PEG + 4.7 Kn	0.45 2,4-D + 0.03 PEG + 0.5 Kn	White and friable
**45 2,4-D + 60 AgNO$_3$ + 0.25 PEG + 4.7 Kn	–	Small, rounded, globular, smooth structures developed
**13.8 spermidine + 1.2 Kn + 1.3 NAA	–	Anthocyanin developed
**13.8 spermidine + 4.5 TDZ + 1.2 Kn + 1.3 NAA	–	Anthocyanin associated with small, shiny, organized structures
**13.8 spermidine + 4.5 TDZ + 1.2 Kn + 1.3 NAA + 2.9 GA$_3$	–	Anthocyanin associated with small, shiny, organized structures developed, green in colour; appeared as developing into shoots, but did not develop further

Culture incubation: 6 weeks.
*Leaf explants used for direct organogenesis.
**White, friable callus used for indirect organogenesis.
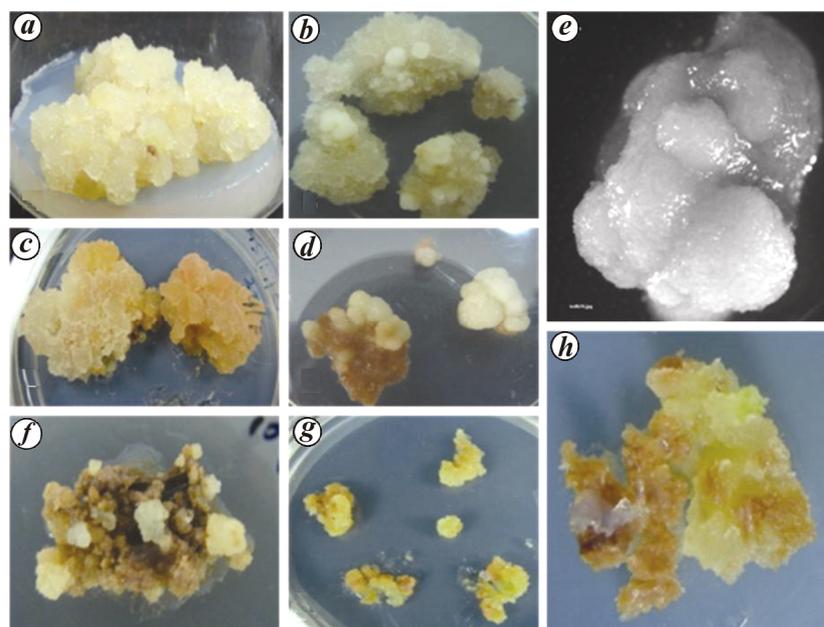

Figure 4. Indirect organogenesis in long subcultured callus grown on 2,4-D. *a*, Callus for organogenesis. *b*, Small, white, shiny, smooth and globular structures on callus with 60 µM AgNO$_3$ and 0.25 µM PEG. *c*, Anthocyanin pigment developed on callus in the presence of 13.8 µM spermidine along with 1.2 µM Kn and 1.3 µM NAA. *d–f*, Small, shiny, organized structures developed in the presence of 13.8 µM spermidine and 4.5 µM TDZ. *g*, *h*, Development of green coloured shoot-like structures in the presence of 2.9 µM GA$_3$.

globular structures appeared only on long subcultured callus at least more than 8 weeks old. AgNO$_3$ was added to the medium because it acts as a direct inhibitor of ethyl-ene action[11], thus enabling delayed senescence of the callus. It has been reported that AgNO$_3$ influences somatic embryo induction and development in *Fraxinus*

mandshurica Rupr[12] and also good growth of the proliferated shoots in rose[13]. PEG treatment was observed to increase the regeneration potential of the callus cultures of sugarcane[14].

Further, the same callus having small white, shiny, smooth and globular structures was subcultured for the development of regenerants in the medium containing 13.8 µM spermidine along with 1.2 µM Kn and 1.3 µM NAA and the cultures were kept in the dark. The callus developed red pigmentation (Figure 4 *c*). In the presence of 13.8 µM spermidine and 4.5 µM TDZ, this red callus developed small shiny organized structures (Figure 4 *d–f*). It has been already reported that the appearance of any pigment in the callus is a marker for the onset of organogenesis. The deposition of phenolic compounds in cotton[15], synthesis of β-carotene in carrot[16], and flavonoids in *Iphiona mucronata*[17] also appeared before organogenesis. It has been observed[13] that efficacy of cytokinins could be enhanced when used in combination with GA_3. We used 2.9 µM GA_3 for further growth of these structures, following the report by Gonbad *et al.*[18]. These shiny organized structures developed green structures resembling shoot buds (Figure 4 *g, h*) but could not develop further into shoots. In *W. mirabilis*, there is no earlier report other than that of Bornman and Fanshawe[7,8], who used different sucrose concentrations (2–8%) and stress factors by increasing culture temperature and nutrient agar concentration, respectively, to study the structural organization and response of callus. They could not observe organogenesis, although chlorophyll content increased with the increase in sucrose concentration. Large numbers of tracheary elements were formed with lower concentration of sucrose, but without organogenesis. Due to the effect of stress, the cells were roughly spherical and small, whereas osmotic potential was high. Tracheary elements were both scattered and organized into vascular nodules and sheets. The effects of elevated temperature and agar concentration were probably enacted through an increase in the negative water potential of the cells. Alternatively, high temperature might have an inductive effect, particularly on enzymes implicated in xylogenesis.

Conclusion and future scope

W. mirabilis is an intractable-to-regenerate plant. Tissue culture studies on this long lived plant would be useful in the future for identification of genes related to ageing. To obtain direct or indirect organogenesis, leaf segments from a 26-year-old plant were collected and callus could be induced only from the tip portion. The induced friable callus is being maintained in an optimized medium containing 9.0 µM 2,4-D, 4.4 µM BA, 4.6 µM Kn and 5.4 µM NAA. Small, shiny, smooth, green, and globular structures were found to develop with the use of various plant growth regulators. However, despite our efforts, the structures could not be developed into shoots.

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